

primers were added to the cDNA to generate a 5' EcoRI compatible cDNA end, and an XhoI compatible 3' cDNA end, to facilitate forced orientation cloning into  $\lambda$ ZAP II uni bacteriophage vector. Recombinant bacteriophages were plated out and amplified on *E. coli* XL1-Blue *mrf'*. Total primary clones numbered 800000 with 6% wild type representation.

## 2. Screening with pre-operative antisera:

The cDNA bacteriophage library was plated out of NZY agar plates and the  $\beta$ -galactosidase operon induced using IPTG. Expressed fusion proteins were then transferred to hybond-C membranes (Amersham) and the membranes were then screened with pre-operation antisera from the patient. The antisera used has been described (Rowe *et al.*, 1996). Prior to use the antisera was extensively pre-absorbed with *E. coli* lysate, and whole blood to reduce signal to noise. Rabbit antisera raised against patient BD pre-operation serum (Rowe, Bone 18 (1996), 159-169), was extensively pre-absorbed with normal human serum and *E. coli* lysate in order to remove *E. coli* antibodies and background human-serum derived antibodies. Briefly, five 80 mm diameter nitrocellulose filters were added to whole *E. coli* lysate (Stratagene), and a second set of five filters were soaked with normal human serum (10 ml). The impregnated filters were each incubated for 10 min at room temperature in sequence with 250 ml of 1:1000 diluted anti rabbit pre-operation antisera in 1% BSA; 20 mM Tris-HCl (pH7.5), 150 mM NaCl (TBS); 0.02%  $\text{NaN}_3$ . The preabsorbed pre-operation antisera (pre-Aanti-op) was then used to screen the cDNA library. Bacteriophage  $\lambda$ ZAP II uni OHO cDNA-clones were plated out on *E. coli* XL1-Blue *mrf'* and incubated for 3 hours at 37°C. Hybond N<sup>+</sup> filters preincubated with 10 mM IPTG were then placed on top of the developing plaques and incubated a further 3 h at 42°C. Filters were then removed and washed with TBS supplemented with Tween 20 (TBST), and then blocked with 1% BSA in TBS with 0.02%  $\text{NaN}_3$  overnight at 4°C. Pre-Aanti-op was then added to the blocked filters and left for 1 h at room temperature. Subsequent washes of the filters and incubation with goat-anti-rabbit alkaline phosphatase conjugate, followed by visualization using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium was as described by Stratagenes picoblue™

immunoscreening kit. After screening ~600,000 clones, nine positives were selected and purified by secondary and tertiary screening. The bacteriophage clones were rescued as phagemids using ExAssist helper phage and cloned into *E. coli* SOLR cells. ExAssist helper phage and SOLR cells were purchased from Stratagene Ltd., Suite 140, Cambridge Science Park, Milton Road, Cambridge, CB4 4GF, United Kingdom.

### 3. Sequencing clone:

Phagemids were prepared and the DNA sequenced. All nine clones were sequenced. Positive bacteriophage-plaques were removed from agarose plates after tertiary screening with a sterile hollow quill. The agarose plugs containing the lytic plaques was then added to 0.5 ml of SM buffer supplemented with 0.02% chloroform, and left at 4°C overnight. Rescue and transformation of bacteriophage clones to BSCPT SKI<sup>-</sup> phagemids was carried out using ExAssist phage as described by Stratagene. The host cells for the purified phagemid were *E. coli* SOLR cells. Plasmid DNA was then prepared using standard techniques (Rowe, Nucleic Acids Res. 22 (1994), 5134-5136), and sequenced using ABI fluorescent automated sequencing and standard vector specific primers. Six of the clones were overlapping and in frame with the bacterial  $\beta$ -galactosidase promoter to give contiguous/overlapping epitopes and expressed proteins with identical overlapping DNA sequences. The longest sequenced clone encompassed the cDNA sequences of the five others and is shown in Figure 8. This sequence (amino acid/cDNA) is a complete sequence for phosphatonin. There are 430 amino acid residues cloned (SEQ ID NO: 2) and 1655 bp of DNA sequence (SEQ ID NO: 1). Secondary structure prediction indicates a highly hydrophilic protein with glycosylation at the COOH end, and the presence of a cell attachment tripeptide at the amino end (RGD), see Figure 8. The protein is also highly antigenic with a number of major helical domains (Figure 10). Extensive screening of all available databases using BLAST has not revealed any statistically relevant homology to known genes or protein sequences.

#### 4. Purification of recombinant human phosphatonin:

The isolated cDNA clone is represented as rescued phagemids in Bscpt SKII - vector (Stratagene vector), and contained within SOLR E. coli host cells. Low level fusion protein expression via induction of the  $\beta$ -galactosidase promoter by IPTG has been achieved. The phosphatonin clone fusion-product reacts with pre-operation antisera on western blots. Increased expression and bioactivity of the fusion proteins can be achieved by sub-cloning into the pCAL-n-EK vector (Stratagene vector) (see below). The construct containing human phosphatonin is contained in E. coli (BL21 (DE3) pLysS) cells (purchased from Stratagene). IPTG induction of fusion protein is much higher, and essentially pure protein can be obtained by calmodulin affinity-chromatography of cell lysates. Recombinant phosphatonin with fusion-tag binds to the calmodulin resin in the presence of  $\text{Ca}^{2+}$ . Phosphatonin fusion protein is then released after washing with EGTA. The small microbial fusion-tag is removed by treatment with enterokinase, leaving pure human phosphatonin.

##### 4a. Subcloning Phosphatonin into pCAL-n-EK vector

The entire deduced cDNA coding sequence (deduced from the largest cDNA clone pOH011.1), of phosphatonin (MEPE) was subcloned into the prokaryote expression vector plasmid pCAL-n-EK (Stratagene vector), and the construct transformed into E. coli BL21 (DE3) pLysS and E. coli XL1-Blue mrf' respectively (strains obtained from Stratagene). The method of ligation independent cloning (LIC) was used as described by Stratagene Affinity™ cloning and protein purification kit (cat No: #214405 and #214407). Two primers were designed from the phosphatonin sequence 5' and 3' end respectively with additional overhang linker sequence as follows (bold sequence represents linker):

Forward 5' **GACGACGACAAG**.GTGAATAAAGAATATAGTATCAGTAA 3'

Linker

(SEQ ID NO: 8)

Reverse 5' **GGAACAAGACCCGT**.CTAGTCACCATCGCTCTCACT 3'

Linker

(SEQ ID NO: 9)